In vivo visualization of albumin degradation in the proximal tubule

Craig Slattery[1], Aven Lee[1], Yuan Zhang[2], Darren J. Kelly[2], Peter Thorn[1], David J. Nikolic-Paterson[3,4], Greg H. Tesch[3,4] and Philip Poronnik[1]

[1]School of Biomedical Sciences, The University of Queensland, St Lucia, Queensland, Australia; [2]Department of Medicine, St Vincent's Hospital, University of Melbourne, Melbourne, Australia; [3]Department of Nephrology, Monash Medical Center, Monash University, Clayton, Victoria, Australia and [4]Department of Medicine, Monash Medical Center, Monash University, Clayton, Victoria, Australia

Albuminuria is a key marker of renal injury and a major risk factor for cardiovascular disease. *In vivo* imaging techniques with fluorescent albumin have allowed visualization of its movement within the whole kidney but they could not distinguish between intact and degraded albumin.

To visualize albumin degradation in proximal tubular cells *in vivo* we used an albumin conjugate (dye quenched (DQ)-albumin), which only fluoresces when it is degraded. In cultured proximal tubule cells, the fluorescent signal from DQ-albumin was dependent on endocytosis and lysosomal function and showed that at any time about 40% of endocytosed DQ-albumin was degraded. Significant accumulation of conventional Texas Red-labeled albumin and degraded DQ-albumin was found in rat proximal tubules 5 min after injection. Importantly, no hint of DQ-albumin was detected in the serum, suggesting that the fluorescent signal in the proximal tubules was derived from tubular degradation of intact albumin. Our study shows that DQ-albumin, together with conventional fluorescent conjugates of intact albumin, can be used to visualize albumin degradation by proximal tubules *in vivo*.

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Correspondence: *Philip Poronnik, School of Biomedical Sciences, The University of Queensland, St Lucia, Queensland 4072, Australia. E-mail: p.poronnik@uq.edu.au*

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Albuminuria is an important marker of renal injury. Even low levels of urinary albumin excretion (microalbuminuria) is an independent risk factor for the development of cardiovascular disease.[1] Thus, a detailed understanding of the molecular basis of renal albumin handling will provide fundamental insights into the development and progression of proteinuric renal disease and cardiovascular disease.

The traditional model for renal albumin handling is that the glomerulus acts as a charge and size selective barrier such that in the normal kidney, only small amounts of albumin enter the tubule.[2] This filtered albumin in then reabsorbed by proximal tubular cells by endocytosis and subsequently degraded in lysosomes to its constituent amino acids so that only a trace amount of intact albumin is excreted in the final urine (2–20 mg/day in humans). The increased urinary excretion of immune-reactive albumin that defines micro-albuminuria is thought to be because of breakdown of the glomerular barrier, with the glomerulus becoming 'leaky' for plasma albumin, and the increased levels of filtered albumin saturate the capacity of the tubular endocytic pathway. This also points to a potential role for dysregulation of tubular endocytosis or lysosomal function in the pathogenesis of microalbuminuria.

Considerable research over the last decade has focused on the dynamics of receptor-mediated albumin endocytosis and the molecular mechanisms underlying this process have been described in some detail (reviewed in Refs. 2,3). Many studies in both cultured proximal tubule cells and *in vivo* have used fluorescent analogues of albumin to visualize the movement of albumin following its uptake. More recently, *in vivo* imaging techniques using confocal and two-photon microscopy have been used to visualize albumin uptake by the proximal tubule in intact kidneys in live animals.[4,5] These studies have generated considerable debate by proposing a model in which glomeruli filter significant amounts of albumin and that the proximal tubule reabsorbs most of this filtered albumin intact to the blood by a high-capacity transcytotic retrieval pathway.[3,6] Thus the delineation between degradative and retrieval pathways is of prime importance. However, little experimental data exist regarding the dynamics of albumin degradation in the proximal tubule

and how this pathway is affected by pathophysiological conditions or disease. The kidney is known to have the highest lysosomal content of any tissue.[7] The potential importance of lysosomal degradation of filtered albumin is highlighted by a small number of studies that report a pronounced inhibition of albumin degradation in diabetes.[8,9] Previously, it has not been possible to distinguish between fluorescence arising from intact versus fragmented albumin because the conjugated albumin molecule has several fluorophores attached. The ability to visualize the spatial distribution of degraded and intact albumin in the intact kidney would enable a new understanding of renal albumin handling in normal and diseased kidneys. In this study, we describe and validate the use of autoquenching (dye quenched (DQ)) fluorescent albumin conjugates as experimental tools to investigate the degradation of albumin in the proximal tubule both *in vitro* and *in vivo*.

RESULTS

Validation of DQ-albumin as a probe

DQ-bovine serum albumin (hereafter called DQ-albumin; Molecular Probes, Eugene, OR, USA) is a bovine serum albumin conjugate labeled to such a high degree with either red or green BODIPY dyes that the dye is selfquenching and therefore displays no detectable fluorescence. In other words, intact DQ-albumin does not fluoresce. However, upon proteolysis the quenching effect is abolished, resulting in highly fluorescent albumin fragments.[10] In our initial experiments using a fluorescence microplate reader, we confirmed that intact DQ-albumin displayed no detectable fluorescence. Tryptic digestion of DQ-albumin resulted in a significant level of fluorescence that saturated with time (Figure 1a) and was not affected by pH (Figure 1b). Polyacrylamide gel electrophoresis was also performed on the DQ-albumin exposed to trypsin. As expected, this resulted in a progressive degradation of the albumin probe with associated laddering observed on the gels with a strong band appearing at the dye front, which corresponds to small peptide fragments (Figure 1c). Fluorescent gel imaging demonstrated the removal of autoquenching after trypsinization. Given that the main aim of this study was to validate the use of DQ-albumin *in vivo*, a critical control was to verify that there was no significant degradation of the DQ-albumin during the time that it was transiting in the blood. Therefore, we collected serum from rats that had been injected with a bolus of DQ-albumin for 5 min (see *in vivo* studies below for

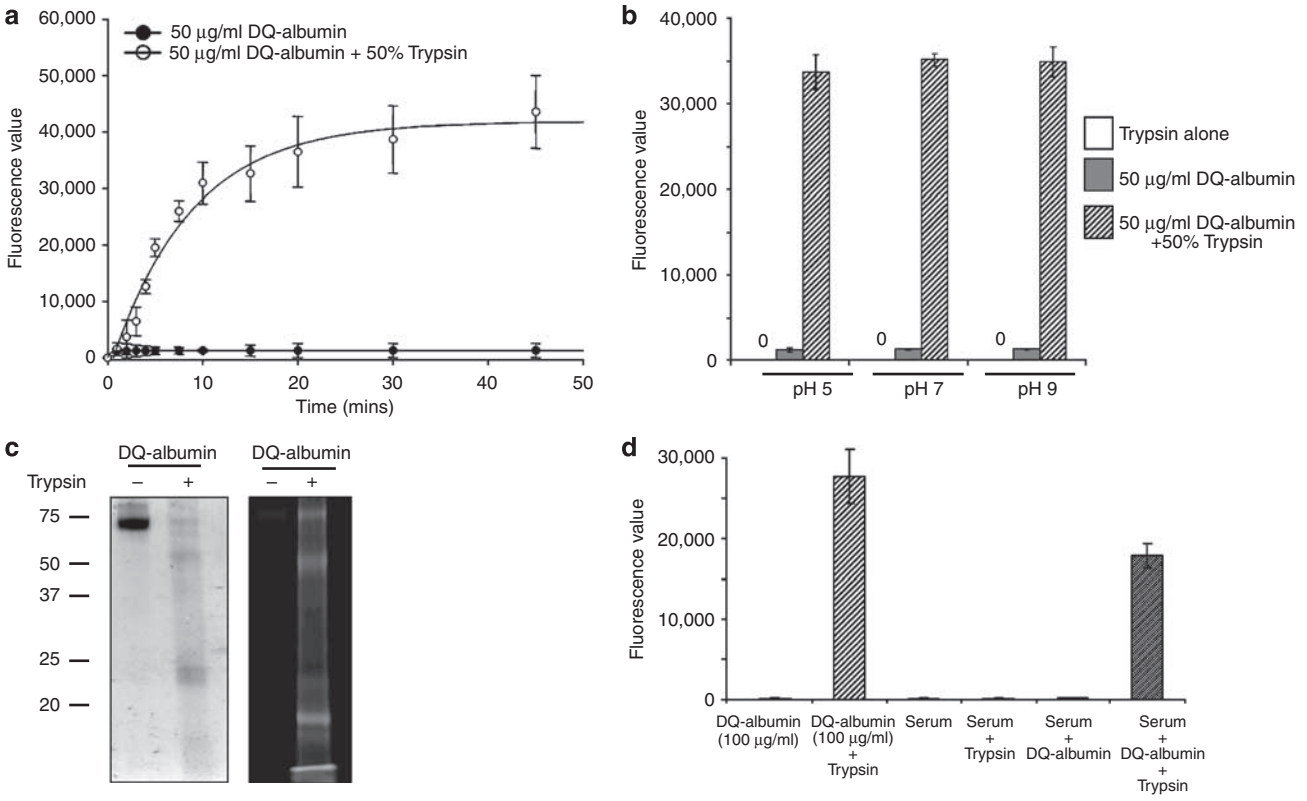

Figure 1 | Characterization of DQ-albumin. DQ-albumin fluorescence was measured using a BMG Fluostar Optima at 550 nm excitation and 620 nm emission wavelengths. (**a**) DQ-albumin (50 μg/ml) was exposed to trypsin for 1 h. Fluorescence was measured at the indicated time points. (**b**) DQ-albumin was exposed to trypsin at pH 5, 7, or 9 for 5 min at which point fluorescence was measured. (**c**) SDS–PAGE analysis of DQ-albumin conjugates in the absence and presence of trypsin. Protein bands were visualized by Coomassie staining whereas fluorescent bands were visualized using a Typhoon fluorescent imager. (**d**) Rat serum was analyzed before DQ-albumin injection and 5 min after DQ-albumin injection. Samples were incubated in the absence and presence of trypsin for 5 min at which time fluorescence was measured. Error bars represent the mean ± s.e.m. of at least three independent experiments.

details). Importantly, we were unable to detect any signal in the serum; however, when the serum was treated with trypsin, an immediate strong fluorescent signal was observed (Figure 1d).

DQ-albumin fluorescence in cell culture

The opossum kidney (OK) proximal tubule cell line is widely used to study albumin uptake *in vitro*. We used OK cells to determine the extent to which DQ-albumin could be used to visualize albumin degradation. OK cells were incubated with increasing concentrations of DQ-albumin (20–2,000 μg/ml). DQ-albumin accumulation within OK cells was saturable with maximum fluorescence occurring at 720 ± 82 μg/ml and half-maximal at 75 ± 12 μg/ml (Figure 2a). It has been reported previously that unlabeled albumin can displace fluorescently labeled albumin.[11] Similarly, we found that the DQ-albumin signal was decreased in the presence of unlabeled albumin in a concentration dependent manner (Figure 2b). We have previously reported that albumin uptake is effectively abolished by disruption of the actin cytoskeleton.[12] Similarly, we found that treatment of OK cells with latrunculin A (2 μM) resulted in a significant inhibition of albumin degradation as shown by the reduction in DQ-albumin fluorescence (38 ± 9.1%; $n = 3$; $P < 0.01$; Figure 2c). Incubation with chloroquine (100 μM), an inhibitor of lysosomal acidification, also caused a reduction in DQ-albumin fluorescence (88 ± 7%; $n = 3$; $P < 0.001$; Figure 2c). Thus, the characteristics of the uptake and fluorescence of DQ-albumin are remarkably similar to those of conventional Texas Red-labeled albumin (TR-albumin). One key remaining question is what percentage of the endocytosed albumin is actually degraded? To answer this question, OK cells were incubated with DQ-albumin (50 μg/ml) for up to 180 min. The cells were washed and the cell lysates divided into equal aliquots. One aliquot was untreated as a measure of cellular degradation of the DQ-albumin whereas the other was incubated with trypsin to measure the maximum DQ-albumin fluorescence in each sample. The signal in both aliquots was measured and DQ-albumin fluorescence was expressed as a percentage of total DQ-albumin fluorescence. Across all time points, degradation of DQ-albumin ranged from 37 to 45% ($n = 5$) of the total DQ-albumin amount taken up into the cells (Figure 2d).

We next used confocal and two-photon microscopy to determine the spatial distribution of conventional Alexa-conjugated albumin with that of DQ-albumin. OK cells were incubated with Alexa-albumin (25 μg/ml) and DQ-albumin (25 μg/ml) for 2 h at 37 °C and then fixed. Confocal microscopy revealed that the Alexa-albumin probe was widely distributed in the cells (Figure 3ai). In contrast,

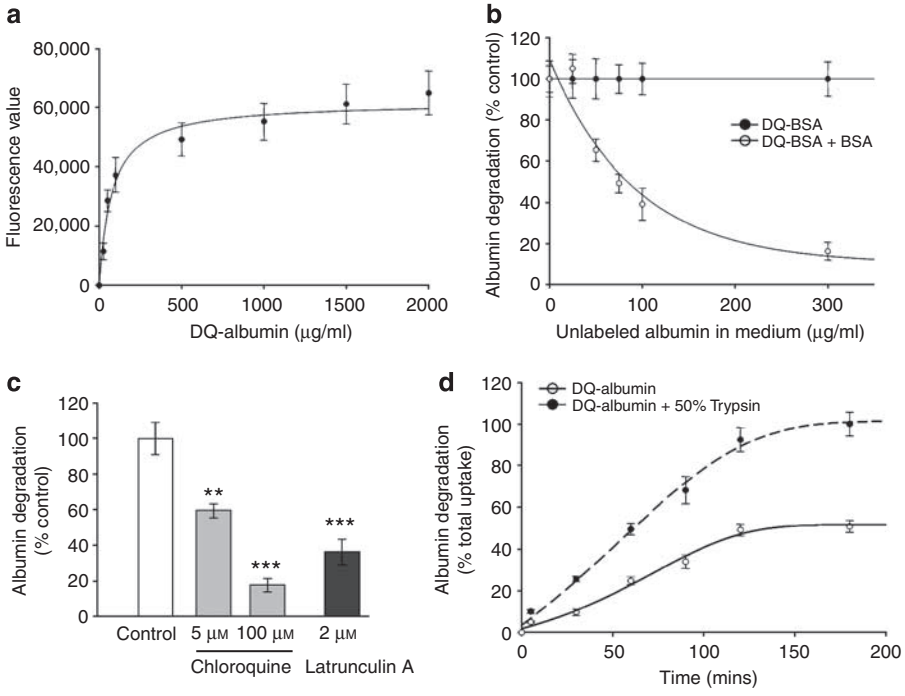

Figure 2 | DQ-albumin in proximal tubule epithelial cells. DQ-albumin fluorescence was measured using a BMG Fluostar Optima at 550 nm excitation and 620 nm emission wavelengths. (**a**) OK cells were exposed to increasing amounts of DQ-albumin (20–2,000 μg/ml) for 2 h, at which time DQ-albumin fluorescence in OK cell lysates was measured ($n = 5$). (**b**) OK cells were exposed to DQ-albumin (50 μg/ml) in the presence of increasing concentrations of unlabeled albumin (0–300 μg/ml) for 2 h, at which time DQ-albumin fluorescence in OK cell lysates was measured. (**c**) OK cells were exposed to DQ-albumin (50 μg/ml) in the absence and presence of chloroquine (5 or 100 μM) or latrunculin A (2 μM) for 2 h, at which time DQ-albumin fluorescence in OK cell lysates was measured. (**d**) OK cells were incubated with DQ-albumin (50 μg/ml) for up to 180 min. Cells lysates were divided into equal aliquots. One aliquot was untreated, whereas the other was incubated with trypsin before measuring fluorescence. The DQ-albumin signal in the untreated samples was expressed as a percentage of trypsinized (total) DQ-albumin fluorescence. Error bars represent the mean ± s.e.m. of at least three independent experiments. **($P < 0.01$) and ***($P < 0.001$) indicate statistically significant difference compared to control.

DQ-albumin was mainly concentrated in larger vesicular structures at the basolateral aspect of the cells (Figure 3aii). Size analysis with ImageJ software revealed that the average size of the DQ-containing vesicles was significantly larger ($43 \pm 11\%$; $n = 177$; $P < 0.01$) than those containing Alexa-albumin. Distribution analysis showed a pronounced presence of Alexa-albumin containing vesicles at the most apical regions of the cells. In contrast, DQ-albumin vesicles were not detected in these regions but were detected 1.5–2 μm below the most apically located Alexa-albumin vesicles (data not shown). This is consistent with DQ-albumin fluorescing at a later stage of the endocytic pathway. With each probe there was no saturation of the signal and no spectral overlap was detected. As expected, because the Alexa-albumin is also being degraded and the fragments that fluoresce are also present in this subcellular location, merging of the two signals revealed significant colocalization of the two probes in the DQ-labeled structures (Figure 3aiii). An advantage of two-photon microscopy over confocal microscopy is that the image is not contaminated by fluorescence out of the focal plane, thereby allowing a high degree of spatial resolution. We next used two-photon microscopy with a lysosomal marker (Lysosensor, Molecular Probes) to confirm that the DQ-signal was associated with the degradative pathway. The Lysosensor dyes are acidotrophic probes that accumulate in acid vesicles. The images shown in Figure 3b clearly show that the fluorescence from DQ-albumin colocalized with Lysosensor confirming that liberation of the fluorescent signal from DQ-albumin occurs within the degradative pathway.

Visualizing albumin degradation *in vivo* in the kidney

Having validated the use of DQ-albumin as a probe to measure tubular cell uptake and degradation of albumin *in vitro*, we next investigated the uptake and degradation of albumin in the rat kidney. Intravenous TR-albumin or DQ-albumin was administered to rats by bolus injection (10 μg/g body weight) into the tail vein. After a period of 5 min the animals were killed and the kidneys removed immediately for fixation and subsequent processing for confocal microscopy. TR-albumin labeling of proximal tubules was clearly evident 5 min after intravenous injection (Figure 4a–g). TR-albumin containing vesicles were distributed throughout the proximal tubular cells, radiating from the tubular lumen and extending to the basal surfaces of the tubules. In the case of DQ-albumin, a strong signal was also observed in the proximal

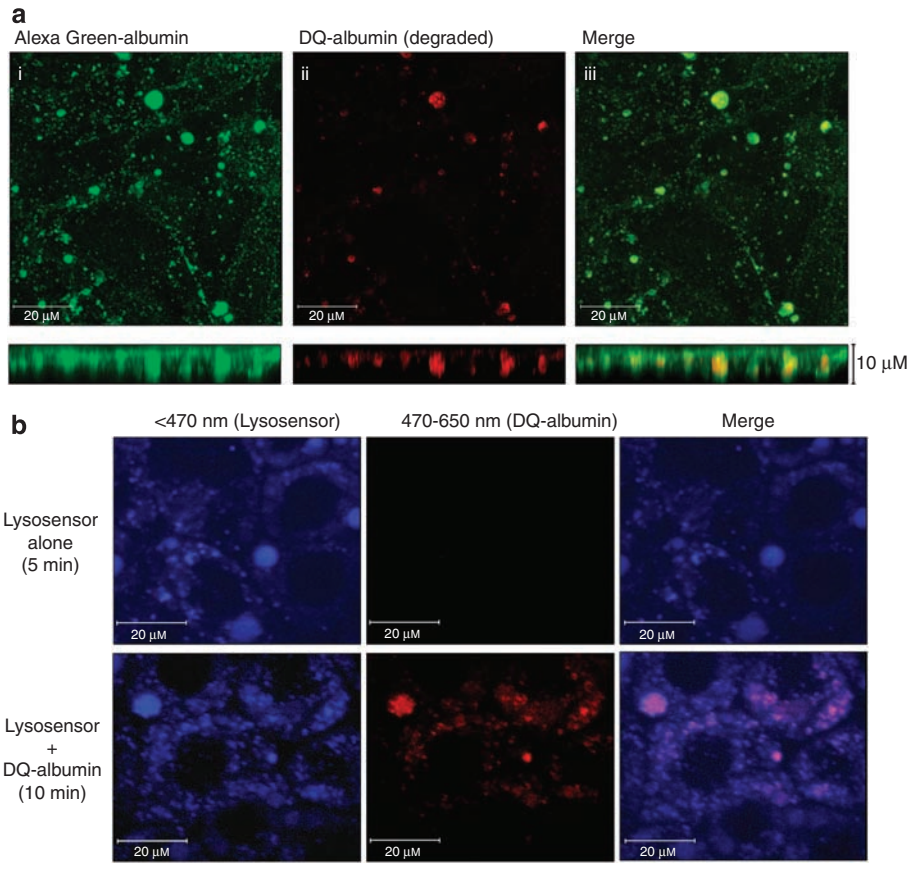

Figure 3 | Microscopic analysis of albumin endocytosis and degradation *in vitro*. (**a**) OK cells were incubated with Alexa-albumin (25 μg/ml) and DQ-albumin (25 μg/ml) for 2 h at 37 °C and then fixed. Fluorescent albumin distribution was assessed by confocal microscopy. (**b**) OK cells were incubated with Lysosensor (1 μM) for 5 min, at which time DQ-albumin (50 μg/ml) was added for a further 10 min. Lysosensor and DQ-albumin localization were assessed in live cells by two-photon microscopy. Images shown are representative of at least four independent experiments.

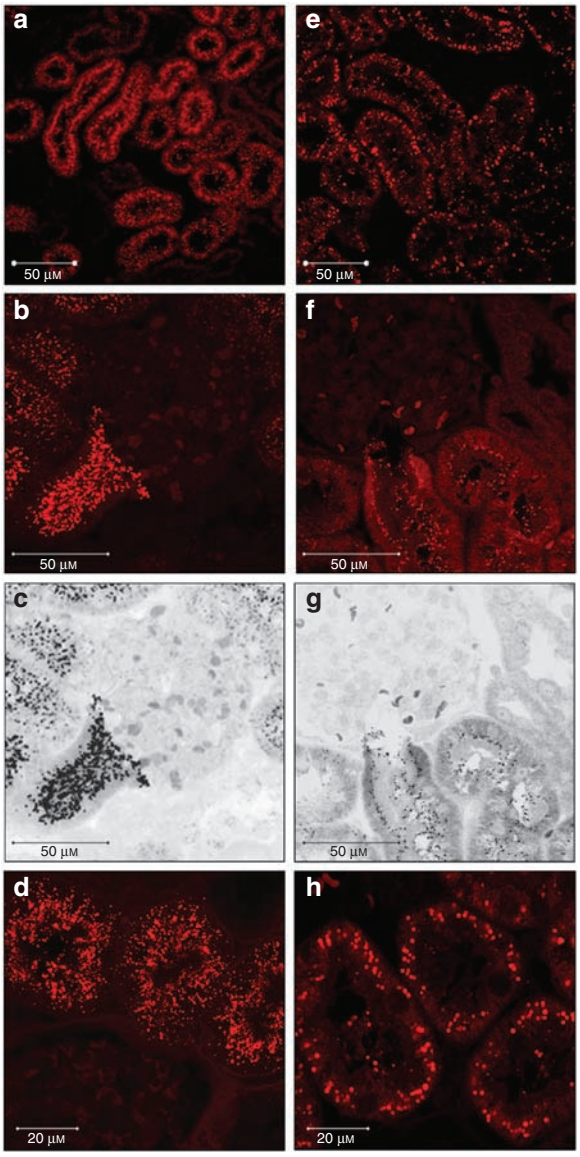

Figure 4 | Confocal microscopic analysis of albumin endocytosis and degradation *in vivo*. Anaesthetized male Wistar rats were injected intravenously with 10 μg/g body weight of TR-albumin or DQ-albumin in 0.5 ml of saline. After 5 min the animals were killed and the kidneys were removed. Kidneys were fixed in neutral buffered formalin, paraffin embedded, and sectioned at 10 μm. TR-albumin (panels **a**–**d**) and DQ-albumin (panels **e**–**h**) were visualized by confocal microscopy. (**a**) TR-albumin uptake within 5 min in proximal tubular cells. (**b**) Image of a single glomerulus with the early proximal tubule leading from Bowman's capsule containing vesicles of TR-albumin. (**c**) Black and white image of the field in (**b**) clearly showing the selective localization of TR-albumin to the proximal tubule. (**d**) High-power view showing a widespread apical to basolateral distribution of TR-albumin in proximal tubules (the adjacent glomerulus is negative for TR-albumin). (**e**) After 5 min of infusion, DQ-albumin fluorescence is evident in larger vesicles that are predominant in the basolateral domain. (**f**) Image of a single glomerulus with the early proximal tubule leading from Bowman's capsule containing vesicles of degraded (DQ-albumin). Note absence of DQ-albumin signal in other tubular cross sections. (**g**) Black and white image of the field in (**f**). (**h**) High-power view showing DQ-albumin fluorescence predominantly at the basolateral domain of the proximal tubules. Images shown are representative of at least four independent experiments.

tubules 5 min after intravenous injection. The fluorescence was primarily in large vesicles, which in most tubules were predominantly localized towards the basolateral aspect of the cells with relatively little signal observed at the brush border. Significantly, in rats injected with a similar concentration of a small molecular weight fluid phase marker sulforhodamine B (the equivalent of free fluorescent probe) we could observe no fluorescence in the tissue sections. This indicates that the signal observed in the TR- and DQ-albumin-injected rats is not because of free probe. The signal could also not be derived from degraded DQ-albumin fragments because we showed that the DQ-albumin was not degraded in the serum (c.f. Figure 1d above). As one further control, we performed a purification step on the Alexa-albumin obtained from Molecular Probes using Microcon YM-50 (Millipore, Billerica, MA, USA) columns to remove any fragments under 50 kDa. Subsequent intravenous injection of this material gave a fluorescence signal in tubules indistinguishable to that seen with the standard purified Alexa-albumin preparation, ruling out potential contamination with labeled albumin fragments (data not shown).

DISCUSSION

Fluorescent albumin conjugates have been used for a number of years to assess the albumin pathway as a whole, however, a consolidated and unified description of how albuminuria develops and how it relates to the progression of renal disease has been problematic. The development of powerful imaging tools such as intravital two-photon microscopy has opened a new approach to explore renal albumin handling. One technical drawback is that it is difficult to correlate the uptake of conjugated albumin with its degradation. The only methods currently available involve indirect measures of albumin degradation, such as size-based analysis of radio-labeled albumin in urine and serum samples,[8] which provide only total measures of albumin degradation and no information at a cellular level.

In this study, we show that DQ-albumin probes can be used to visualize and measure albumin degradation. Our *in vitro* findings demonstrated that DQ-albumin fluorescence is dependent on proteolytic digestion and displays saturable kinetics. The observation that the DQ-albumin signal was reduced in the presence of unlabeled albumin is similar to that previously reported for fluorescein isothiocyanate-albumin.[11] and indicates that DQ-albumin is being handled by the same pathways as for normal albumin. In a cellular context, DQ-albumin degradation in proximal tubular epithelial cells was reduced by inhibition of lysosomal acidification. The fact that all the DQ-albumin fluorescence effectively colocalized with Lysosensor is important as it shows that the DQ-albumin can only be visualized in acid compartments. These compartments are distinct from the early endocytic structures that are found directly associated with the luminal surface that are seen with normal albumin probes. At any given time point during albumin uptake in cultured cells, approximately 40% of the albumin is

degraded, an observation that reflects the high capacity of the proximal tubule to absorb and degrade albumin. This characterization of the properties of DQ-albumin extend previous studies performed in cultured fibroblasts and dendritic cells.[13,14]

Most importantly, we demonstrated that DQ-albumin is suitable as an *in vivo* probe to visualize albumin degradation. We could detect no DQ-fluorescence in the serum of the injected rats indicating that over 5 min, there was no significant degradation of the albumin as it circulated through the animal. These data also argue that TR-albumin remains intact in the circulation during the experimental time course. Furthermore, we performed an additional purification step on the conventional Alexa-albumin to rule out the possibility of contamination by lower molecular weight fragments. Thus, the fluorescence that we detect in the proximal tubules must originate from intact albumin that has crossed the glomerulus.

We observed readily detectable levels of TR-albumin distributed throughout proximal tubule epithelial cells 5 min after intravenous injection. The significant DQ-albumin fluorescent signal seen in tubules 5 min after administration demonstrated that not only is there rapid uptake of filtered albumin, but that the albumin is also rapidly degraded. This is consistent with the data from cultured proximal tubule cells. The distribution of the TR-albumin is also similar to that reported by Ohno *et al.*[5] who used micropuncture and confocal imaging to track the intracellular vesicular trafficking of albumin in the intact proximal tubule. This study is the first visualization of renal degradation of albumin and shows that not only are appreciable levels of albumin rapidly reabsorbed from the glomerular filtrate, but also that the degradation of albumin occurs with the same temporal profile. However, the approaches used in this study are not able to temporally dissociate albumin uptake and degradation from any potential transcytotic pathway as proposed by Russo *et al.*[6]

In summary, because of technical limitations and different methodologies, it remains a challenge to combine and reconcile data regarding the molecular basis of albumin handling by the proximal tubule and the changes that occur in disease. This study demonstrates the utility of using DQ-albumin conjugates to visualize albumin degradation in the intact kidney. Importantly, this method significantly increases the power of interpreting data obtained with conventional fluorescent albumin conjugates to enable differentiation between cellular compartments containing intact and degraded albumin. This may be a powerful tool with which to examine alterations in tubular uptake and degradation of albumin in diabetic nephropathy and other proteinuric diseases.

MATERIALS AND METHODS

Reagents

DQ, Alexa488, and TR conjugates of bovine serum albumin, and Lysosensor (blue/yellow) were obtained from Invitrogen (Carlsbad, CA, USA). Latrunculin and chloroquine were obtained from Sigma (St. Louis, MO, USA). All other reagents were of the highest available purity from commercial sources.

Cell culture

The opossum proximal tubular epithelial cell line (OK; obtained from Dr D Markovich, University of Queensland) was maintained as previously described and albumin assays were performed under serum free conditions.[12,15,16]

Albumin degradation assay

An established method for measuring albumin endocytosis in OK cells was adapted.[12,15,16] DQ-albumin fluorescence was determined using a BMG Fluostar Optima (BMG Lab Technologies, Offenburg, Germany) at 550 nm excitation and 620 nm emission wavelengths. DQ-albumin degradation was adjusted for background and normalized for total cellular protein.

Confocal and two-photon analysis of albumin degradation *in vitro*

OK cells were seeded on glass coverslips, grown to confluency and serum deprived. OK cells were exposed to Alexa488-albumin (25 μg/ml) and DQ-albumin (25 μg/ml) for 1 h. Cells were fixed and then analyzed by confocal microscopy (Zeiss LSM 510 Meta confocal microscope using $\times 40$, $\times 63$, and $\times 100$ objectives). Z-scan optical sections were collected at 0.2 μM intervals. Alexa488-albumin was excited at 488 nm and emission was measured at 515 nm. TR-albumin and DQ-Red-albumin were excited at 543 nm, and emission was measured at 570 nm. For Lysosensor imaging, a custom built two-photon microscope was utilized[17] ($\times 40$ objective). OK cells were seeded on glass coverslips, grown to confluence and serum deprived. Live imaging was performed and OK cells were exposed to Lysosensor (1 μM) for 5 min followed by exposure to DQ-albumin (50 μg/ml). Image processing and analysis was performed using ImageJ (Rasband, WS, ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2005). Statistical significance was determined using analysis of variance on GraphPad Prism (San Diego, CA, USA).

Animal studies

Anaesthetized male Wistar rats (200–250 g; University of Queensland Biological Resources) were injected intravenously with 10 μg/g body weight of TR-albumin or DQ-albumin in 0.5 ml of saline or with 0.5 ml saline alone. After 5 min, a blood sample was collected, kidneys were removed and the animals killed. Kidneys were fixed in neutral buffered formalin, paraffin embedded and sectioned at 10 μm.

DISCLOSURE

All the authors declared no competing interests.

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